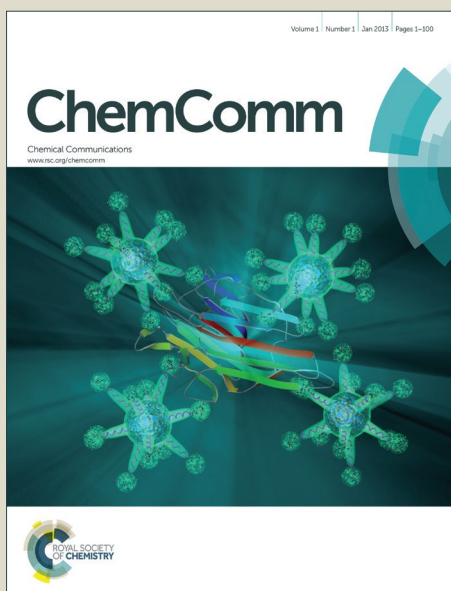


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ARTICLE TYPE

A simple fluorescence off-on probe for the discrimination of cysteine from glutathione†

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A simple and stable fluorescence off-on probe for discrimination of cysteine (Cys) from glutathione (GSH) has been developed by combining resorufin with 7-nitrobenzofurazan. The probe, displaying distinct emission patterns for Cys and GSH at just one excitation wavelength, can be used for simultaneous determination of Cys and GSH in human plasma.

Cysteine (Cys) and glutathione (GSH), two main thiol compounds with low molecular weight in biosystems, play important roles in several diseases such as cystinosis, liver damage, and cancer.¹⁻³ Cysteine is a precursor not only for many proteins but also for GSH, and GSH levels are often used to evaluate the redox and detoxification status in organisms due to its protective ability against oxidative and free-radical-mediated injury.⁴ Therefore, it is of great importance to develop an efficient method for the simultaneous detection of Cys and GSH in clinical samples.

Toward this end, fluorescence measurement has attracted much attention because of its simplicity as well as high selectivity and sensitivity, and till now a number of fluorescent probes have been developed to detect Cys and GSH by using different chemical reactions.⁵⁻¹⁸ Among them, the most often used one is based on the Cys-induced nucleophilic aromatic substitution-rearrangement reaction. However, disadvantages such as low quantum yield, relatively short analytical wavelength, poor stability or water solubility still exist in the existing probes. In particular, with these probes (except the cyanine-based but unstable probe) fluorescence measurements usually have to be performed at two sets of excitation wavelengths, which not only makes the analytical operation inconvenient but also increases measurement errors. Thus, new stable fluorescent probes are still desired for the simultaneous determination of Cys and GSH with simple measurement operation. Herein, we develop such a fluorescence off-on probe (**1**; Fig. 1) for this purpose by combining resorufin with NBD through an ether bond. The probe exhibits high stability, and distinct emission patterns for Cys and GSH at just one excitation wavelength, which leads to the establishment of a simple and practical method for the simultaneous detection of Cys and GSH in clinical samples such as human plasma.

As shown in Fig. 1, probe **1** can be readily prepared by coupling resorufin with NBD through an ether bond. We chose the two fluorophores of resorufin and NBD on the basis of the

following considerations: first, resorufin has good water solubility, high quantum yield, and especially a rather broad excitation spectrum in the wavelength range of 450-575 nm,^{19,20} which would greatly benefit the choice of single excitation wavelength in fluorescence measurements. Second, NBD possesses a unique property of nucleophilic aromatic substitution-rearrangement to Cys instead of GSH.²¹⁻²³ This would result in the formation of different reaction products for Cys and GSH (Fig. 1), thereby showing distinct spectroscopic signals and achieving the discrimination of the two thiols.

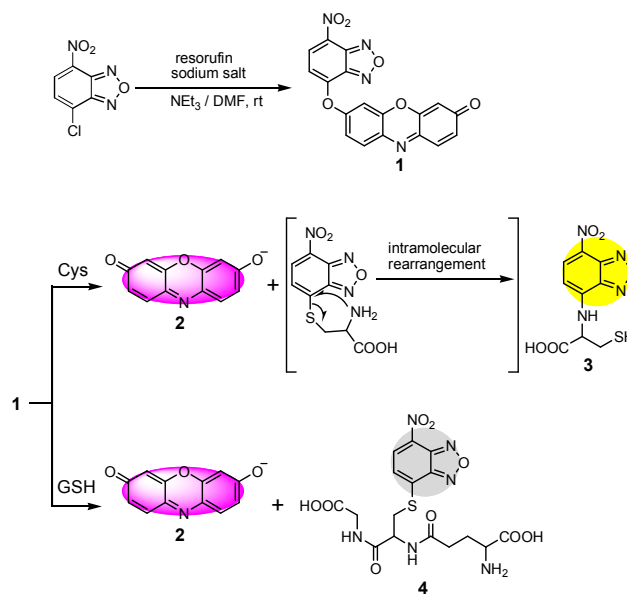


Fig. 1 Synthesis of probe **1** and its possible reaction with Cys and GSH.

The spectroscopic properties of the probe were first investigated in details (Figs. 2 and 3; see also Fig. S3 in ESI†). As shown in Fig. 2, the probe itself is almost colourless, but its reaction solution with Cys or GSH shows pink colour (the inset) with a strong absorption peak at 570 nm, indicative of the release of free resorufin. This was confirmed by mass spectral analysis ($m/z = 212$ [M]⁺; Fig. S4 in ESI†). Notably, the reaction products of **1** with Cys also give an additional shoulder peak at about 475 nm, which is ascribed to the formation of **3** from the Cys-induced nucleophilic aromatic substitution-rearrangement ($m/z = 283$ [M]⁺; Fig. S4A). The formation of **3** was further verified by using 3-

mercaptopropionic acid as a model compound, which lacks the amino group (as compared to Cys) and thus can not form an amino-substitution product (Fig. S5A in ESI†). On the other hand, because GSH also lacks the proximal amine group required for the intramolecular rearrangement, its reaction product (**4**; $m/z = 469$ [M]⁻ in Fig. S4B) from the NBD moiety of **1** keeps the direct connection of the S atom (strong fluorescence quenching ability) to the NBD skeleton (Fig. S5B), which causes a rather weak absorption at 425 nm.

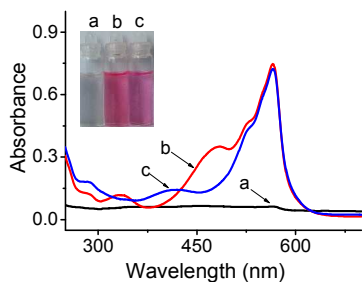


Fig. 2 Absorption spectra of **1** (10 μ M) before (a) and after reaction with 150 μ M of Cys (b) or GSH (c) at 25 $^{\circ}$ C for 10 min. The inset shows the corresponding colour change of **1** before and after reaction.

Fluorescence excitation spectra of **1** reacting with Cys and GSH are shown in Fig. S3 (ESI†). For Cys, two fluorescent products, which were characterized to be **2** and **3** as noted above, display the excitation maxima at 570 nm and 470 nm (Fig. S3A), respectively; for GSH, only one excitation maximum at 570 nm is observed (Fig. S3B), which is ascribed to the formation of the single fluorescent product of **2** (Fig. S4B). To assure a considerable fluorescence generation from both of resorufin and **3**, and thus to achieve the simultaneous detection of Cys and GSH at single excitation wavelength, fluorescence measurements would be made better in the wavelength range of 450–520 nm (Fig. S3A). Obviously, the usage of the 470-nm excitation maximum from **3** would lead to the highest sensitivity.

Fig. 3 shows fluorescence emission spectra of **1** reacting with varied concentrations of Cys and GSH by using the single excitation wavelength of 470 nm. As is seen, the probe alone is non-fluorescent (quantum yield²⁴ $\Phi \approx 0.003$), but its reaction with Cys or GSH generates large fluorescence enhancement. Notably, different emission behaviours were observed for Cys and GSH, in which Cys produces two emissions at 540 nm and 585 nm while GSH generates only one emission at 585 nm. Thus, Cys can be easily identified by the simultaneous appearance of the two emissions, whereas GSH can be characterized by the generation of the single emission at 585 nm. These phenomena, consistent with those from absorption spectra, also support the formation of different products between Cys and GSH. Namely, Cys induced the generation of **2** and **3**, which are responsible to the emissions at 585 nm and 540 nm, respectively; whereas GSH resulted in the formation of one fluorescent product (**2**) and one non-fluorescent product (**4**), the former giving the characteristic emission of resorufin at 585 nm and the latter hardly showing fluorescence due to the strong quenching ability of the S atom. The above different emission patterns at a single excitation provide the basis to conveniently discriminate Cys from GSH.

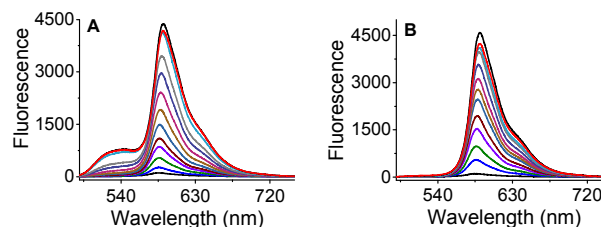


Fig. 3. Fluorescence response of **1** (10 μ M) to Cys (A) and GSH (B) at varied concentrations (from bottom to top: 0, 2, 5, 9, 12, 18, 25, 35, 50, 70, 90, 200 and 500 μ M). The reaction was conducted for 10 min in 10 mM PBS (pH 7.4) at 25 $^{\circ}$ C. $\lambda_{\text{ex}} = 470$ nm.

Next, effects of pH, reaction temperature and time were studied to optimize the conditions for Cys and GSH assay. As shown in Fig. S6A (ESI†), Cys shows the strongest fluorescence in neutral media, but GSH scarcely produces fluorescence in the range of pH 4.8–9.4 tested, indicating that, if Cys and GSH coexist, Cys can be accurately determined at $\lambda_{\text{em}} = 540$ nm without the interference from GSH at a near neutral pH value of 7.4. Interestingly, the fluorescence of **1** reacting with GSH also reaches a near plateau in the media of pH 7.4, at which the assay of GSH can thus be performed by using an emission wavelength of $\lambda_{\text{em}} = 585$ nm. On the other hand, the fluorescence response from Cys rather resembles that from GSH, which indicates that in the presence of Cys the quantification of GSH may be achieved by subtracting the fluorescence contribution of Cys at 585 nm because of fluorescence additivity. Change of temperature from 25 $^{\circ}$ C to 45 $^{\circ}$ C has little effect on the fluorescence of the probe reacting with Cys and GSH (Fig. S7). Thus, room temperature of 25 $^{\circ}$ C was used in our experiment. Moreover, the fluorescence intensity increases to a maximum in about 5 min upon addition of Cys or GSH (Fig. S8), indicating a fast reaction of the probe with the two thiols. Importantly, the fluorescence from **1** and its reaction solution keeps unchanged for at least 6 h, demonstrating high stability of the present detection system.

Then, the linearity of fluorescence response of **1** to Cys and GSH was studied, disclosing the increase in fluorescence intensity of **1** with the increase of the Cys/GSH concentration from 0 to 500 μ M. For Cys, an about 86-fold fluorescence enhancement at 540 nm and an about 41-fold at 585 nm were observed, whereas for GSH an about 43-fold fluorescence increase was produced at 585 nm only. Moreover, good linear equations were acquired. For Cys, two linear equations were obtained: one being $\Delta F = 5.57 \times C (\mu\text{M}) - 17.2$ ($R = 0.997$) at 540 nm in the range of 3.1 – 90 μ M (Fig. S9A), and the other being $\Delta F = 61.05 \times C (\mu\text{M}) + 160.8$ ($R = 0.989$) at 585 nm in the range of 1 – 40 μ M (Fig. S9B). For GSH, only one linear equation of $\Delta F = 125.2 \times C (\mu\text{M}) + 193.0$ ($R = 0.990$) was obtained at 585 nm in the range of 1 – 18 μ M (Fig. S9C). The detection limit ($S/N = 3$) at 585 nm was 0.13 μ M and 0.07 μ M for Cys and GSH, respectively.

Various potential interfering species, such as amino acids (Gln, Ala, Asp, Ser, Arg, Hcy), inorganic salts (MgCl_2 , CaCl_2 , CuSO_4 , NaNO_2 , NaNO_3), and other biomolecules (creatinine, uric acid, glucose and ascorbic acid) were examined to explore the specificity of **1** for Cys and GSH. As shown in Fig. S10, **1** shows good selectivity for Cys and GSH over the other species tested

except homocysteine (Hcy). Hcy exhibits a similar fluorescence response to Cys due to their structural similarity. However, the concentration of Hcy is at least ten times lower than that of Cys in normal plasma,^{25,26} therefore the effect of Hcy could be ignored in the analysis (Fig. S11). These results suggest that probe **1** may be capable of selectively detecting Cys or GSH in clinical samples.

Although there are some fluorescent probes reported for discriminative detection of Cys and GSH, none of them has been applied to the simultaneous determination of Cys and GSH in human plasmas. Here we have made such an attempt by using our probe. Prior to measurements, the fluorescence additivity of the present detection system was verified by using different mixture samples of Cys with varied concentrations of GSH in the linear range. As shown in Fig. S12, the fluorescence spectra measured from the mixture samples accord well with the additive fluorescence spectra obtained through the theoretical calculation (error < 2%), clearly indicating that, if Cys and GSH coexist, Cys can be accurately determined at $\lambda_{em} = 540$ nm first, and then GSH is quantified by subtracting the fluorescence contribution from Cys at 585 nm. In this way, human plasma samples, diluted appropriately to fit the linear detection requirement, were analyzed. As shown in Table 1, the average concentration of Cys in human plasmas is about 289 μ M, and that of GSH is about 29 μ M, which are in agreement with the reported results (130 to 290 μ M Cys, and 5 to 50 μ M GSH in human plasmas).^{3,25-28} Furthermore, different concentrations of Cys and GSH were added to the plasmas, and the recovery by the present method was determined. As is seen from Table 1, the recoveries of Cys and GSH range from 95 to 109% and from 102 to 104%, respectively, with a relative error of no more than 7%, suggesting that the proposed method is of great potential for discriminative determination of Cys and GSH.

Table 1 Determination of Cys and GSH in human plasmas

Analyte in plasmas	added (μ M)	found ^a (μ M)	Recovery ^a (%)
Cys	0	289.4 \pm 3.6	—
	200	479.6 \pm 12.0	95 \pm 6.0
	400	727.0 \pm 9.0	109 \pm 2.2
GSH	0	29.0 \pm 1.6	—
	25	54.8 \pm 1.4	104 \pm 6.4
	50	80.2 \pm 3.4	102 \pm 3.0

^a Mean of three determinations \pm standard deviation

In summary, by combining resorufin with 7-nitrobenzofurazan, we have developed a new fluorescent probe for the discriminative determination of Cys and GSH. The probe is rather stable, and displays a fast but distinct fluorescence off-on response to Cys and GSH at only one excitation wavelength, which makes it convenient to simultaneously determine Cys and GSH, and such a capability of the probe has been demonstrated for the measurement of Cys and GSH in human plasmas.

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- † Electronic Supplementary Information (ESI) available: apparatus and reagents, and other supporting data. See DOI: 10.1039/b000000x/
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